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(54) Title: THE USE OF HIV–1 INTEGRASE IN SCREENING HIV–1 DRUG CANDIDATES

(57) Abstract

The present invention harnesses the roles of IN to screen drug candidates to inhibit HIV–1. The present invention uses IN mutants and fusion proteins to assess the effectiveness of a drug candidate in inhibiting a native IN function. The retrovirus integrase protein (IN) is essential for integration of the viral DNA into host cell chromosomes as well as for retroviruses in general. The ability of a drug candidate to inhibit retroviral replication utilizes a monitoring system such as a host cell culture infected with a retrovirus. After exposing the monitoring system to the drug candidate, the retroviral life cycle within the monitoring system is monitored and compared to monitoring system life cycle function of integrase mutant retrovirus. Alternatively, an integrase fusion protein of either vpr or vpx is added to a monitoring system infected with a retrovirus expressing wild–type integrase following exposure to a drug candidate. Changes in retrovirus life cycle function are monitored for suppression of a wild–type integrase function indicating retroviral replication has been comprised.

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THE USE OF HIV-1 INTEGRASE IN SCREENING HIV-1 DRUG CANDIDATES

Field of the Invention

The present invention relates to the role of integrase (IN) in the retroviral life cycle and biological systems that require IN function including those for the screening of drug candidates and gene therapy for interfering with native IN function and thus inhibiting retroviral replication and especially HIV-1 replication.

Background of the Invention

The cocktail treatments currently given to HIV patients are not completely successful in long term suppression of HIV. The high dosages, variety of active substances and requirements of the treatment regimen all indicate that the cocktail does not fully inhibit the HIV life cycle. Thus, there exists a need to find an additional target.

The retroviral integrase (IN) protein catalyzes integration of the HIV provirus and is essential for persistence of the infected state in vivo. The protein structure and the biochemical mechanism of the catalytic integration reaction of IN are known (5, 14, 30). HIV-1 IN is expressed and assembled into the virus particle as a part of a larger 160 kDa Gag-Pol precursor polyprotein ($Pr160^{Gag\text{-}Pol}$) that contains other Gag (matrix, capsid, nucleocapsid, p6) and Pol (protease, reverse transcriptase, integrase) components. After assembly, $Pr160^{Gag\text{-}Pol}$ is proteolytically processed by the viral protease to liberate the individual Gag and Pol components, including the 32 kDa IN protein. Recent studies on IN function using replicating virus (in vivo) have suggested that in addition to catalyzing integration of the viral cDNA, IN may have other effects on virus replication (23,

35, 41). *IN* mutations can affect virus replication at multiple levels, for instance, mutations in *IN* affect the Gag-Pol precursor protein and alter assembly, maturation, and other subsequent viral events and also affect the mature IN protein and its organization within the virus particle and the nucleoprotein/preintegration complex. Therefore, such mutations are pleiotropic and may alter virus replication through various mechanisms, and at different stages in the virus life cycle. At least in part, this likely explains the diverse phenotypes that have been reported for IN mutant viruses. HIV-1 is representative of the class of retroviruses in terms of the function of IN. These have included viruses with defects in assembly, virion morphology, reverse transcription, nuclear import, and integration of the provirus (3, 7, 16, 44, 46). While it is obvious that a full understanding of IN function requires analysis in higher-ordered systems that accurately reproduce both the viral and host-cell environments, the pleiotropic nature of IN mutations has complicated such studies, and thus, there remains a significant gap in our understanding of IN function in vivo.

Numerous in vitro studies have examined the biochemical and genetic properties of retroviral IN proteins, and provided most of the information for the currently accepted mechanism of the integration reaction. Using purified IN and oligonucleotides that represent the viral DNA ends, the in vitro integration reaction proceeds in two steps: IN removes two nucleotides from the 3' terminus of the viral DNA (terminal cleavage), which is then joined to a break in the cellular DNA (strand transfer) (6, 22, 43). Through amino acid sequence alignment and in vitro activity studies of wild-type and mutant IN proteins,

distinct functional domains have been identified that are conserved among retroviruses (12, 15, 33, 53). In the case of HIV-1, the N-terminal domain (residues 1-50) contains a highly conserved HHCC motif. Mutation of this motif has variable effects on 3' processing and strand transfer and its function remains poorly understood (15, 36, 51, 52). The central region (residues 51-212) contains the invariant acidic residues D64, D116, and E152. Mutation of any of these residues causes a loss of all IN activity in vitro, suggesting that this region is the catalytic center of the enzyme (15, 36, 51). The carboxyl terminal region (residues 213-288) is least conserved and possesses nonspecific DNA binding properties. Certain mutations within the C-terminal region may not significantly effect the activity of IN in vitro, but cause a dramatic loss of virus infectivity (10, 16, 56).

Reverse transcription is catalyzed by the reverse transcriptase (RT), and although reverse transcription can occur in vitro using recombinant RT, template and primer, the process is more intricate in vivo. In the context of a replicating virus, complete synthesis of the viral cDNA is not as simple as putting together different proteins and nucleic acids, rather, it is a multi-step process involving a number of transitional structures. Within the infected cell, reverse transcription takes place in the context of a nucleic acid-protein (nucleoprotein) complex that includes other viral and cellular factors (7, 18, 19, 29, 42). Moreover, synthesis of the viral cDNA is greatly dependent on the proper execution of numerous molecular events that precede reverse transcription. In the case of HIV-1, several viral regulatory proteins are known to affect reverse transcription. Nef mutant viruses exhibit a 5- to 50-fold reduction in DNA synthesis (2, 45). Pseudotyping

with VSV-G complements this defect, indicating that Nef affects uncoating, and in turn reverse transcription (1). Vif mutant viruses produced by primary cells (nonpermissive) are defective in viral DNA synthesis (47, 54). It remains unknown whether this is due to a direct or indirect effect of Vif on reverse transcription (9, 38). Tat has been shown to be required for efficient reverse transcription in infected cells (25). The nucleocapsid protein, which facilitates strand transfer, may also increase the efficiency of reverse transcription (24, 37). Mutations of the critical proline residues within the capsid domain of p55Gag, which are important for binding cyclophilin A, result in virions that enter cells normally but fail to initiate viral DNA synthesis (4, 21, 40, 49). Taken together, these results have shown that reverse transcription can be effected by multiple factors and at various levels of the virus life cycle. In the absence of a detailed understanding of the molecular mechanisms involved in the formation of infectious virus and the structure and composition of the reverse transcription complex, it is difficult to differentiate between factors that are specific and directly affect reverse transcription versus those that involve other steps in virus replication, such as assembly, maturation and uncoating.

Most in vivo studies of IN function have utilized virus derived from IN mutant proviral DNA, where detection of an integrated provirus was the primary marker for IN activity. Since mutations in the HIV-1 IN gene can cause defects in virus replication prior to integration, assays that rely on integration are not always useful for dissecting the function of IN at the virus replication level. By monitoring for products of reverse transcription in infected cells, certain HIV-1

IN mutants have been found to be defective at or prior to viral DNA synthesis. IN deletion-mutant virus or those with mutations in the HHCC motif of IN have been shown to produce 10- to 20-fold less viral DNA following infection (16, 17, 35, 41). Although normal in proteolytic processing, virion protein composition, encapsidation of the genomic RNA, and virion associated RT activity, it has remained unknown at what level(s) such IN mutations affect the virus life cycle (35, 41). The production of infectious retroviral particles occurs through a highly coordinated sequence of events, and even subtle changes in this process affect events early in the virus life cycle, such as reverse transcription. The sensitivity of viral DNA synthesis to events that occur earlier is reflected by the high proportion of virions that are unable to initiate reverse transcription.

To directly analyze the function of the mature IN protein itself, HIV accessory proteins (Vpr or Vpx) are utilized as vehicles to incorporate other proteins into HIV virions by their expression in trans as fusion proteins (39, 57-59). Expression as fusion partners of Vpr or VPX demonstrate that fully functional RT and IN are efficiently incorporated into HIV-1 particles independently of the Gag-Pol precursor protein (39, 57). Moreover, virions derived from an RT and IN minus proviral clone are infectious and replicated through a complete cycle of infection when complemented in trans with RT and IN fusion proteins. Thus, it is possible to decouple the function of the mature IN protein from that of the Gag-Pol precursor protein. The present invention results from directly analyzing IN protein function by introducing mutations into IN without interfering with Gag-Pol function and other late stage events that could

affect reverse transcription. It is known that the defect in the replication of various IN mutant viruses could be complemented by the Vpr-IN fusion protein (20, 39). However, prior efforts did not address the specific nature of the defect or the mechanism by which the trans-IN protein was able to complement the impaired phenotype, nor did these studies contemplate a utility for the defect or mechanism to screen for drugs effective against an IN function.

In discovering additional roles of IN in the HIV life cycle, it becomes apparent that there exists a need for methods that use this IN function including those that may be used for screening drug candidates that interferes with a HIV life cycle function or delivering gene therapy by way of coupling with IN.

Summary of the Invention

The present invention provides a method for drug discovery effective against retroviral replication by way of exposing a potential drug candidate through a monitoring system such as a host cell culture which has been infected with a retrovirus. Thereafter, a particular retrovirus life cycle function is monitored following exposure of the monitoring system to the drug candidate. Thereafter, the retrovirus life cycle function is optionally compared with that of a retrovirus integrase mutant life cycle function within the same type of monitoring system infected with an integrase mutant retrovirus. The present invention also provides a method for discovering drugs effective against retroviral replication including exposing a potential drug candidate to a host cell culture infected with a retrovirus. Thereafter, a particular retrovirus life cycle function is monitored following exposure of the host cell culture to the drug candidate. An

integrase fusion protein is then introduced to the host cell culture and the changes associated with the retroviral life cycle function are assessed before and after introduction of the integrase fusion protein. An integrase fusion protein also serves as a model of IN function independent of a retroviral infected host culture. A method of the present invention is practiced using a commercial package including an integrase fusion protein or an integrase mutant retrovirus nucleotide sequence.

Description of the Drawings

Figure 1 shows an agarose gel of DNA products amplified from wild-type ($pSG3^{wt}$) and mutant (S-IN, H12A, H16A, F185A, Δ22, D116A, S-RT and D443N) proviral clones.

Figure 2(A) shows the immunoblot analysis of Vpr-IN complemented virions. Wild-type and mutant (S-IN, H12A, H16A, F185A, Δ22, D116A, S-RT and D443N) proviral DNA clones, respectively, are individually transfected (-) or cotransfected (+) into 293T cells with the pLR2P-vprIN expression plasmid.

Figure 2(B) shows an agarose gel of DNA products amplified from wild-type ($pSG3^{wt}$) and mutant (S-IN, H12A, H16A, F185A, Δ22, D116A, S-RT and D443N) proviral clones after Vpr-IN expression in trans with each of the mutant viruses.

Figure 3(A) shows the immunoblot analysis of $pSG3^{S\text{-}RT}$ transfection (-) or cotransfected (+) with 293T cells with the Vpr-RT, Vpr-$^{\Delta PC}$IN and Vpr-RT-IN expression vectors in 293T cells using anti-RT, anti-IN, anti-Vpr and anti-Gag antibodies indicators.

Figure 3(B) shows an agarose gel of amplified DNA products of wild-type and mutant viruses of Figure 3(A) from HeLa-CD4 cells. DNA products of reverse transcription are prepared and analyzed as described in Example 5.

Figure 4(A) shows an agarose gel demonstrating that enzymatically defective trans-IN protein supports reverse transcription in IN mutant proviral clones by comparing U5-gag production for transfected mutant and cotransfected mutant with Vpr-IN^{D116A} and Vpr-IN vectors, respectively.

Figure 4(B) is a bar graph showing integration frequency of mutant expression vectors cotransfected with Hy-SG3^{D116A} and Hy-SG3^{D116A} transfected with the control vector pLR2P.

Figure 5(A) is an agarose gel showing that HIV-2IN (IN^2) does not efficiently support HIV-1 reverse transcription.

Figure 5(B) is a bar graph showing integration frequency of mutant expression vectors Vpr-IN and Vpr-IN^2 cotransfected with Hy-SG3^{AA35A} and Hy-SG3^{AA35A} transfected with pLR2P as a control.

Detailed Description of the Invention

The present invention utilizes the expression and incorporation of functional IN protein into virions in trans to analyze at what stage in the virus life cycle different IN mutations affect retrovirus reverse transcription and demonstrates that the mature IN protein itself is required for viral DNA synthesis in vivo.

By comparing the life cycle behavior of a wild type HIV-1 IN in the presence of a drug candidate to that of mutant IN HIV-1, the efficacy of the drug

candidate is assessed by the present invention. The present invention utilizes IN fusion protein effectiveness in activating HIV-1 function in the presence of a drug candidate upon expression in trans to elucidate the mechanism by which the candidate suppresses IN protein function.

Since function IN is essential to the formation of the RT complex, delivery of a gene therapy agent in conjunction with IN represents a novel method of insuring transgene expression upon retroviral DNA synthesis. A gene therapy transgene readily transforms a host genome through transgene sequence placement adjacent to IN coding sequences of a proviral clone. The transgene illustratively encodes for surface proteins recognizable by a host immune system, an apoptosis gene and the like.

As used herein, the term "monitoring system" is defined as a medium in which a retrovirus or mutant thereof carries out a viral life cycle function, and illustratively includes a cell culture, a cellular component extract solution, and a liposomal or lipid bilayer structure having receptor proteins thereon for a retrovirus or mutant thereof.

As used herein, the term "complex" is defined as the association of retroviral constituent polypeptides, proteins or nucleic acid sequences necessary for DNA synthesis mediated by IN.

As used herein, the term "drug candidate" is defined as a molecular species that potentially disrupts DNA synthesis through retroviral RT complex formation and includes organic molecules, organometallic molecules, nucleic acid sequences and amino-acid sequences.

As used herein, the term "retrovirus" is defined as an RNA virus belonging to retroviridae and illustratively includes simian immunodeficiency virus (SIV), HIV-1, and HIV-2.

While the specification is largely directed towards the representative retrovirus HIV-1, similar results are also obtainable for other retroviruses including SIV and HIV-2. Therefore, it is appreciated that the present invention has utility for other retroviral IN life cycle functions including as a method for drug discovery against IN function within the general class of retroviruses and by this mechanism inhibits retroviral replication.

Certain IN mutant viruses are impaired in reverse transcription. Different IN mutant viruses are generated and characterized for their ability to synthesize viral DNA. These included S-IN (IN minus), H12A and H16A, (disturbs the conserved HHCC motif located in the N-terminus), F185A (structurally positioned near the catalytic center), Δ22 (deletes 22 amino acids from the C-terminus), and D116A (destroys enzymatic activity). HeLa-CD4 cells are infected with 500 nanograms of each virus, and analyzed eighteen hours later for the presence of early (R-U5), intermediate (U3-U5), and late (R-gag) DNA products of reverse transcription. Ten- to twenty-fold less early (R-U5) DNA is detected in cells infected with all of the IN mutant viruses, with the exception of D116A as shown in Figure 1, lanes 2-8. The DNA products of Figure 1 are obtained and characterized according to the procedure of Example 5. Similar changes are detected for the intermediate and late DNA products. No viral DNA is detected in cells that are infected with the control S-RT virus (lacking both RT

and IN, (57). The RNaseH defective RT mutant virus (D443N) produced the early R-U5 DNA product in amounts similar to wild-type virus but the levels of the intermediate and late DNA products are dramatically reduced, indicating that the strong-stop DNA product is stable for at least 18 hours in infected cells. At a four hour time point after infection, the relative proportions of wild-type and IN mutant DNA products are similar to those measured at eighteen hours. The reverse transcription products as detected by PCR are confirmed to have been synthesized within the infected cells, since AZT completely inhibited the detection of mutant and wild-type viral DNA. Table 1 shows that similar concentrations of intracellular CA protein are detected for both the wild-type and mutant viruses, indicating that the impaired DNA synthesis of the IN mutants is not due to a block at the level of virus entry. As shown in Table 1 mutant viruses, with the exception of the S-IN mutant, exhibited normal levels of virion associated RT activity. The analysis for two-LTR circular viral DNA confirmed that the nuclear import of nascent DNA of each IN mutant was not impaired in dividing cells such as HeLa cells.

The HeLa-CD4-LTR-β-gal cell line (32) is used as a biological indicator for a defect in viral DNA synthesis. Table 1 shows that the "infectivity" of the IN mutant viruses was decreased 20- to 100-fold compared to that of the integration defective D116A virus, which supports wild-type levels of viral DNA synthesis. The present invention relies on the data showing that mutations in certain regions of IN impair DNA synthesis in infected cells in order to target IN as a central species in the HIV life cycle.

Trans IN protein restores viral DNA synthesis to IN mutant viruses.

To examine whether changes in the Gag-Pol precursor protein or the IN protein are responsible for the defect in reverse transcription, the Vpr-IN fusion protein is expression in trans with each of the different IN mutant viruses previously detailed. Figure 2(A) confirms that the Vpr-IN fusion protein is efficiently packaged and processed by the viral protease to liberate the mature 32 kDa IN protein. Mutant viruses that contained the Vpr-IN fusion protein ($trans_r$-IN) exhibited a 10- to 20-fold increase in the synthesis of early, intermediate, and late viral DNA products as shown in Figure 2(B). Thus, the Vpr-IN fusion protein, which was assembled into virions together with mutant Gag-Pol precursor protein (S-IN, H12A, H16A, F185A, and Δ22, respectively), restored viral DNA synthesis. The MAGI assay confirmed that Vpr-IN also restored virus infectivity, to levels between 15 and 58% compared with that of wild-type virus as shown in Table 1. While the trans-IN protein complemented viral DNA synthesis and infectivity, it did not correct the defect in Gag processing (excess p39) or virion associated RT-activity as shown in Figure 2(A) and Table 1. Vpx-IN fusion protein ($trans_x$-IN) exhibited the same 10- to 20-fold increase in the synthesis of viral DNA products as $trans_r$-IN (data not shown). While the results for Vpr-IN are shown herein, it is appreciated that other fusion partners to IN are also operative in the present invention to restore IN function to a monitoring system supporting an IN mutant retrovirus. These IN fusion partners illustratively include Vpx, Vpr and fragments thereof capable of delivering IN to a virion; generally such a

fragment includes at least ten sequential amino acid residues of the wild fusion protein partner.

Trans IN protein acts after virus assembly to promote viral DNA synthesis. As shown in Figure 3(A), the RT-IN minus provirus (S-RT) (57) is complemented with the Vpr-RT fusion protein. While high RT activity levels are associated with the progeny virions, the virions remain severely defective in DNA synthesis. However, when complemented with both Vpr-RT and Vpr-IN together or Vpr-RT-IN, viral DNA synthesis is increased 40- to 80-fold compared with Vpr-RT complemented virions, Figure 3(B). Similar results are obtained using Vpx as a fusion partner with IN and RT (data not shown). These results demonstrate that the failure of the IN mutant viruses to support reverse transcription is not due to a defect at the level of $Pr160^{Gag\text{-}Pol}$, but rather, that the mature IN protein is central to retroviral DNA synthesis in vivo.

In parallel with the experiment described above, Figure 3(A) (lane 3) shows that S-RT virions complemented with both Vpr-RT and Vpr-$^{\Delta PC}$IN contained processed RT and minimal amounts of processed Vpr-$^{\Delta PC}$IN. For Vpr-$^{\Delta PC}$IN containing virions, no significant increase is detected in the synthesis of viral DNA compared with S-RT virions that is complemented with only Vpr-RT as shown in Figure 3(B). Since the Vpr-IN and $Vpr^{\Delta PC}$-IN fusion proteins are isogeneic, except for the amino acid substitution at the P1' position of the cleavage site, and both assemble into virions as an uncleaved 47 kDa fusion protein, therefore, free IN protein is necessary for efficient reverse transcription.

Complementation between IN mutants. By incorporating IN mutant proteins Figure 4(A) shows that the integration defective trans$_r$-IN^{D116A} protein restores DNA synthesis to each of the DNA synthesis defective (H12A, H16A, F185A, Δ22) mutant viruses. Each of the S-IN, H12A, H16A, F185A, and Δ22 IN mutants is incorporated as a Vpr-IN-mutant fusion proteins into the D116A mutant virus, which is DNA synthesis positive and integration defective. Figure 4(B) shows that some but not all of the Vpr-IN-mutants were able to rescue viral DNA integration. The trans$_r$-INF^{185A} and trans$_r$-IN$^{\Delta 22}$ mutants markedly increased integration frequency. This result demonstrated that the F185A and Δ22 mutants still possessed the integration activity necessary to catalyze provirus formation in vivo, and that the integration and reverse transcription functions of IN can occur independently. In contrast, the trans$_r$-IN^{H12A} and trans$_r$-IN ^{H16A}mutants did not efficiently support integration, indicating that mutations in the highly conserved HHCC motif disturb both the reverse transcription and integration functions.

Virus type-specific IN is required for efficient viral DNA synthesis. The HIV-2 IN protein (IN^2) is incorporated into IN minus (S-IN) virions by expression as a Vpr-IN^2 fusion protein. Figure 5(A) shows that despite efficient virion incorporation and proteolytic processing of Vpr-IN^2, only a modest (2-3 fold) increase in HIV-1 DNA synthesis is observed compared with a 10-20-fold increase induced by the homologous IN. However, when integration defective mutant virus is complemented with Vpr-IN^2, the integration frequency was increased nearly 100 fold as shown in Figure 5(B). Thus, the HIV-2 IN protein is able to associate with the HIV-1 reverse transcription complex, but that this

alone is not sufficient to support DNA synthesis. This demonstrates that specific interactions between the homologous IN and other viral components of the reverse transcription complex are required to promote viral cDNA synthesis in vivo.

Direct physical interaction between the HIV-1 RT and IN proteins. HIV RT and IN form a heterodimeric complex based on: (i) the two proteins are known to coexist as a complex in some retroviruses (30, 50); (ii) the carboxy terminal domain of RT (RNaseH) and the central core domain of IN are structurally similar (11, 14); and (iii) in murine leukemia virus, IN and RT proteins can be co-immunoprecipitated with antibodies to either protein (27).

After assembly, the structures of the Gag and Gag-Pol precursor polyproteins change due to proteolytic processing. Processing of the Gag and Gag-Pol precursors drives the metamorphosis of the immature (noninfectious) virion into one with a condensed, mature core structure containing the diploid single-stranded viral RNA genome, nucleocapsid (NC), reverse transcriptase, integrase, and primer tRNA. In the early stage of the virus life cycle, after entry into the host cell, the virus core structure undergoes additional rearrangements (uncoating) to form a nucleoprotein complex structure that supports reverse transcription. After reverse transcription is completed, IN catalyzes integration of the nascent viral cDNA into the host cell's chromosomes. The present invention identifies that the mature IN protein itself is required for efficient reverse transcription, independent of its enzymatic function, this is exploited to screen drug candidates targeted to disrupting the reverse transcription function of IN or alternatively the integration function of IN. The IN protein promotes the

initiation step of reverse transcription through virus type-specific interactions with other components that make up the reverse transcription initiation complex.

The IN protein supports viral DNA synthesis after virus assembly and proteolytic processing. It is noteworthy that some mature IN protein is detected in virions complemented with the $Vpr^{\Delta PC}$-IN fusion protein, Figure 3(A), yet the virions remain noninfectious.

The present invention indicates that IN mutant proteins associate with the nucleoprotein/preintegration complex. Figure 4 shows that the $trans_r$ F185A IN mutant, which did not support reverse transcription, restores integration to D116A mutant virions. Moreover, complementation of the IN mutant viruses (F185A, and Δ22) with the $trans_r$-D116A IN mutant ($Vpr\text{-}IN^{D116A}$) restored DNA synthesis and integration (Figure 4). Similarly, the heterologous trans HIV-2 IN protein also efficiently supported integration of HIV-1 DNA, but did not support reverse transcription (Figure 5). The IN protein promotes reverse transcription through virus specific (not cellular) interactions with other viral components in the reverse transcription complex.

Recent studies have suggested that reverse transcription may be regulated at three defined stages: initiation; transition, the point between initiation and elongation; and elongation (34). The efficiency of initiation requires specific and multiple interactions between viral and cellular components, including the viral RNA genome, RT, NC, and primer tRNA. Also included are interactions of the primer tRNA with the PBS and an A-rich loop located 12-17 nucleotides upstream of the PBS (28, 55). The disturbances of any of these interactions may cause

defects in the initiation of reverse transcription in vivo. Initiation is a slow process and proceeds at a highly reduced processivity compared with elongation (34). Viral DNA elongation shows that for the H12A, H16A, F185A, and Δ22 IN mutant viruses, the minus-strand strong-stop DNA product is produced in similar amounts to that of the intermediate and late DNA products. Also, it was shown that the $trans_r$-IN protein supported the synthesis of minus-strand strong-stop DNA to an extent similar to later DNA products. These results indicate that the IN protein is required either prior to or at the initiation stage of reverse transcription, Table 1. Recent studies with nef and certain gag mutant viruses have shown that defects in uncoating, which impair virus DNA synthesis, can be overcome if the normal virus entry pathway is bypassed via pseudotyping with the VSV-G envelope (1). The present invention shows that VSV-G pseudotyping of IN mutant viruses did not overcome the defect in infectivity, rather that the IN protein is directly involved in reverse transcription in vivo. In the case of the avian retroviruses the IN protein comprises an integral component of the RT heterodimer, an RT-IN polypeptide makes up the beta subunit (30, 50). Thus, the IN protein forms an integral part of the reverse transcription initiation complex and specifically promotes interactions between RT, the genomic RNA and primer $tRNA^{Lys,3}$ that facilitate initiation.

The apparent fragility of the reverse transcription initiation complex (34) and the sensitivity of reverse transcription to mutations in any of the three IN subdomains, show that the DNA synthesis function of IN is particularly vulnerable to anti-IN compounds. Of particular interest is that disturbances in the highly

conserved HHCC motif caused defects in virus replication at two levels, in both reverse transcription and integration (Figure 4). Therefore, drugs which target this motif inhibit virus replication at both levels.

The present invention harnesses the discovery of a role for IN in reverse transcription of retroviruses to screen for drug candidates which are capable of inhibiting retroviral replication through interactions with IN. Typically, a host cell culture is established which is amenable to infection by a retrovirus of interest. The host culture represents a subset of monitoring system for detecting retrovirus life cycle functions such as DNA synthesis and formation of a retroviral RT complex. It is appreciated that the effectiveness of a particular host cell culture as a monitoring system is determined by the selectivity of the retrovirus towards the host cell culture. Upon detection of a monitoring system with a retrovirus or transfection with a retroviral plasmid, a baseline level of retrovirus life cycle function is determined. The subsequent addition of a drug candidate to the monitoring system is then quantified as to its effect on retrovirus life cycle function in comparison to the baseline value. Rather than merely monitoring the effect of a drug candidate on a retrovirus life cycle function, parallel experimentation using the same monitoring system which has been transfected with an integrase mutant retrovirus plasmid serves to elucidate the mechanism of drug candidate interaction with differing portions of the integrase protein. Further, the use of a series of integrase mutant transfected cultures, varying from one another in the location and type of mutation, offers additional streamlining to the drug discovery methodology. A typical methodology of the present invention is

detailed with particularity towards HIV-1 with HIV-1 integrase mutants being used which include: S-IN, H12A, H16A, F185A, Δ22 and D116A.

The present invention also suitably screens drug candidates effective against retroviral replication through targeting of integrase through introduction of a drug candidate to a retrovirus infected monitoring system and monitoring changes in a retrovirus life cycle function such as DNA synthesis or formation of a retroviral RT complex before and after introducing the drug candidate. Provided a drug candidate modifies the retrovirus life cycle function then introduction of an integrase fusion protein into the monitoring system confirms drug candidate activity with integrase upon at least partial restoration of the retroviral life cycle function being monitored. The drug candidate is optionally introduced as a fusion with IN.

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention. It is to be understood that the following is by way of example only and not intended as a limitation on the scope of the invention.

Example 1 - Cells, HIV-1 clones and expression plasmids.

The 293T, HeLa-CD4 and HeLa CD4-LTR/β-gal indicator cell lines (32) are maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U of penicillin and 0.1 mg/ml of streptomycin. The wild-type pSG3^{wt}, RT-IN minus pSG3$^{S\text{-}RT}$, IN minus pSG3$^{S\text{-}IN}$, and IN mutant pSG3^{D116A} proviral clones have been described (38, 57). The RNaseH mutant pSG3^{D443N} proviral clone is constructed by substituting the

aspartic acid residue with an asparagine residue at position 443 of RT, using PCR methods. This mutation inactivated the RNaseH activity of RT. The pHy-SG3 IN^{AA35A} clone is constructed by inserting alanine residues into the hygromycin resistance pHy-SG3 clone (submitted) at each of the three amino acid positions (D64A, D116A, E152A) that comprise the catalytic center of the IN protein. The Vpr-IN, Vpr-RT, Vpr-RT-IN expression vectors were described earlier (38, 57). The Vpr-IN^{H12A} Vpr-IN^{H16A}, Vpr-$^{IN\Delta22}$, Vpr-$^{\Delta PC}$IN are constructed by PCR-based mutagenesis of pLR2P-vpr-IN. The Vpr-$^{\Delta PC}$IN plasmid is constructed to disrupt normal proteolytic cleavage and liberation of IN protein. The leucine residue at P1' position of the RT-IN cleavage site was substituted with an isoleucine residue. The Vpr-IN^{F185A} expression vector is constructed by substituting the F185A mutant IN into pLR2P-vprIN. The Vpr- HIV-2 IN expression plasmid (pLR2P-vprIN^2) is constructed by substituting a BglII/XhoI, IN containing, DNA fragment of $HIV\text{-}2^{ST}$ into pLR2P-vprIN. The PCR amplified IN fragment included 30 base pairs of RT sequence, which is included to preserve the natural protease cleavage site at the N-terminus of IN.

Example 2 - Transfections and virus purification.

DNA transfections are performed on monolayer cultures of 293T cells using the calcium phosphate DNA precipitation method according to the manufacturer's recommendations (Stratagene). Unless otherwise noted, all transfections are preformed with 4 μg of each plasmid. Supernatants from the transfected cultures are collected after forty-eight hours, clarified by low speed centrifugation (1000g, 10 min), and analyzed for RT activity as described

previously (13) and for HIV-1 capsid protein concentration by p24 antigen ELISA (Coulter Inc.). Virions are pelleted by ultracentrifugation through cushions of 20% sucrose using a Beckman SW41 rotor (125,000g, 2 hr).

Example 3 - Semi-quantitative detection of viral DNA.

The PCR technique used to monitor the synthesis of viral DNA in infected cells is similar to those described earlier (4, 41, 54). Briefly, 500 ng equivalents (p24 antigen) of transfection derived virus is used to infect one million HeLa-CD4 cells. To control for variation in virus entry of the different mutant viruses, the intracellular p24 antigen concentration of each virus is determined 4 hours after infection as described earlier (39). At 4 and 18 hours after infection, cells are lysed and total DNA is extracted by organic methods. The DNA extracts are resuspended in 200 µl of distilled water, treated with the DpnI restriction endonuclease to digest bacterially derived plasmid DNA from transfection. The viral cDNA synthesized *de novo* following infection is resistant to cleavage by DpnI. To eliminate any effect of differential virus entry on the detection of viral DNA products in infected cell, the DNA extracts are normalized to 250 pg of p24 antigen for PCR amplification. The wild-type DNA extract is adjusted to 250 pg (100%), 100 pg (40%), 40 pg (16%), 16 pg (6.4%), and 6.4 pg (2.5%). The DNA extracts are then subjected to 30 rounds of PCR amplification using primers designed to detect early (R-U5, [sense nucleotides 1-22: 5'-GGTCTCTCTGGTTAGACCAGA-3' and anti-sense nucleotides 181-157: 5'-CTGCTAGAGATTTTCCACACTGAC-3']), intermediate (U3-U5, [sense nucleotides 8687-8709: 5'-ACACACAAGGCTACTTCCGTGA-3' and anti-sense

nucleotides 181-157: 5'-CTGCTAGAGATTTTCCACACTGAC-3']), and late (R-gag, [sense nucleotides 1-22: 5'-GGTCTCTCTGGTTAGACCAGA-3' and anti-sense nucleotides 355-334: 5'-ATACTGACGCTCTCGCACCCAT-3']), products of reverse transcription, respectively. The PCR products are separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

Example 4 - DNA products of wild and mutant IN clones.

Wild-type ($pSG3^{wt}$) and mutant (S-IN, H12A, H16A, F185A, Δ22, D116A, S-RT, D443N) proviral clones are introduced into 293T cells by calcium phosphate DNA transfection methods. Forty-eight hours later, culture supernatants are filtered through 0.45 micron filters, and analyzed by HIV-1 p24 antigen ELISA (Coulter Inc.). The virus-containing culture supernatants are normalized to 500 ng of p24 antigen (CA), treated with RNase-free DNase H (20 U/ml for 2 hrs., Promega Corp.) and placed on cultures of HeLa-CD4 cells at 37°C. After four hours, the cell monolayers are washed, trypsinized, resuspended in fetal bovine serum and divided into two aliquots. One aliquot set (which contained one-tenth of the total number of cells) is lysed in PBS containing 1% Triton X-100 and analyzed by p24 antigen ELISA to quantify intracellular CA protein, Table 1. The other aliquot set is placed back in culture medium at 37°C for an additional 14 hours. The cells are then washed and total DNA extracted by organic methods. 250 pg equivalents (p24 antigen) of each DNA extract is analyzed by PCR methods for early (R-U5), intermediate (U3-U5), and late (R-gag) viral DNA products of reverse transcription. The amplified products are resolved on 1.5% agarose gels and stained with ethidium bromide as shown in

Figure 1. To assess the relative amount of each of the amplified DNA products, four serial 2.5-fold dilutions of the wild type ($SG3^{wt}$) DNA are analyzed in parallel. The undiluted 250 pg sample is arbitrarily set to 100. As standards, 10 to 6250 copies of the $pSG3^{wt}$ clone are also analyzed by PCR under identical conditions. As a control for the efficiency of DpnI cleavage of potential carryover plasmid DNA, 6250 copies of $pSG3^{wt}$ DNA are analyzed after digestion with DpnI as described (26). The virus origin of the ethidium bromide stained DNA products is confirmed by Southern blot analysis, using a homologous nick-translated probe. The ethidium bromide staining intensity of each amplified DNA produce is measured using a Lynx 5000 molecular biology workstation (Applied Imaging, Santa Clara, CA).

Example 5 - Vpr-IN interaction with IN mutant viruses.

Four micrograms of the wild-type and mutant proviral DNA clones, respectively, are individually transfected (-) or cotransfected (+) into 293T cells with the pLR2P-vprIN expression plasmid. Forty-eight hours later, the culture supernatants are collected, passed through 0.45 micron filters, and analyzed for HIV-1 p24 antigen concentration by ELISA. One-half of the filtered supernatant is centrifuged (125,000g for 2 hours) over cushions of 20% sucrose. The pellets were lysed and examined by immunoblot analysis using anti-IN (top), anti-Vpr (middle) and anti-Gag (bottom) antibodies as described earlier (57) as shown in Figure 2(A). 500 ng of wild-type and each of the mutant viruses is used to infect cultures of HeLa-CD4 cells. After four hours, the cell monolayers are washed, trypsinized, resuspended in fetal bovine serum and divided into two aliquots. One

aliquot set was analyzed by p24 antigen ELISA as described in Example 4. The other aliquot set is placed back in culture medium at 37°C. At 18 hours post infection, the cells are washed and total DNA is extracted by organic methods. The extracts are normalized for intracellular CA protein concentration and analyzed by PCR for viral DNA products of reverse transcription as described in Example 4.

Example 6 - The trans-IN protein functions after virus assembly and proteolytic processing.

Four micrograms of $pSG3^{S\text{-}RT}$ DNA are transfected (-), or cotransfected (+) into 293T cells with the Vpr-RT, Vpr-$^{\Delta PC}$IN, and Vpr-RT-IN expression vectors, respectively. Transfection derived virions are concentrated from the culture supernatants by ultracentrifugation (125,000g for 2 hours) through cushions of 20% sucrose. The pellets are lysed and examined by immunoblot analysis using anti-RT, anti-IN, anti-Vpr and anti-Gag antibodies as indicated in Figure 3(A). 500 ng of transfection derived wild-type and mutant virus are also used to infect cultures of HeLa-CD4 cells. DNA products of reverse transcription were prepared and analyzed exactly as in Example 4 to provide DNA yields.

Example 7 - Complementation between different IN mutants.

Four micrograms of the S-IN, H12A, H16A, F185A, and Δ22 IN mutant proviral clones are transfected alone and separately cotransfected into 293T cells with two micrograms of the Vpr-IN^{D116A} and Vpr-IN expression vectors, respectively. Forty-eight hours later, supernatant virions were prepared and used to infect HeLa-CD4 cells exactly as described in Example 4. The infected cells

are washed eighteen hours later, total DNA is extracted and treated with DpnI endonuclease. The late R-gag DNA product of reverse transcription is PCR amplified and analyzed as described in Example 4 and is shown in Figure 4(A). The D116A IN mutant is constructed into the SG3 hygromycin resistant clone, generating Hy-SG3^{D116A}. The Hy-SG3^{D116A} mutant virus produces wild-type levels of viral DNA, yet is integration defective. Four micrograms of Hy-SG3^{D116A} is transfected with two micrograms of the control vector (pLR2P) and individually cotransfected with two micrograms of each of the Vpr-IN, Vpr-IN^{H12A}, Vpr-IN^{H16A}, Vpr-IN^{F185A}, and Vpr-IN$^{\Delta 22}$ IN mutant expression vectors. Since the env region of Hy-SG3^{D116A} contains the hygromycin marker, the virions are pseudotyped by including the pCMV-VSV-G env vector in the transfection reactions. Forty-eight hours after transfection, the culture supernatants are filtered through 0.45 micron filters and analyzed for HIV-1 p24 antigen concentration by ELISA. Twenty-five nanograms (p24 antigen) of each pseudotyped virus stock is used to infect cultures of HeLa cells. The infected cells are maintained in hygromycin selection medium for 12 days and then stained to identify resistant colonies. The integration frequency is quantified as shown in Figure 4(B).

Example 8 - Analysis of heterologous IN.

Four micrograms of pSG3$^{S\text{-}IN}$ is cotransfected into 293T cells with two micrograms of the Vpr-IN, Vpr-IN^{2} and pLR2P (vector only) expression vectors, respectively. Four micrograms of pSG3^{wt} is also transfected as a control. Forty-eight hours later, supernatant virions are prepared and used to infect HeLa-CD4 cells exactly as described in Example 4. The infected cells are washed eighteen

hours later, total DNA is extracted and treated with DpnI endonuclease. Early (R-U5), and late (R-gag) viral DNA products of reverse transcription are amplified by PCR and analyzed as described in Example 4. To directly compare the ability of the heterologous trans-IN^2 protein with that of the homologous IN protein to support integration of the provirus, the hygromycin resistant, integration defective, Hy-SG3 IN^{AA35A} clone is used for analysis. Hy-SG3 IN^{AA35A} contains a mutation in each of the three residues (D64A, D116A, E152A) that comprise the catalytic center of the IN protein, and efficiently synthesizes viral DNA after entry. Four micrograms of Hy-$SG3^{AA35A}$ is cotransfected with two micrograms each of the Vpr-IN, and Vpr-IN^2 expression plasmids, respectively. The virions are pseudotyped by including the pCMV-VSV-G env vector in the transfection reactions. Forty-eight hours after transfection, the culture supernatants are filtered through 0.45 micron filters and analyzed for HIV-1 p24 antigen concentration by ELISA. Twenty-five nanograms (p24 antigen) of each of the pseudotyped virus stocks are used to infect cultures of HeLa cells. The infected cells are maintained in hygromycin selection medium for 12 days and then stained to identify resistant colonies as previously described. The integration frequencies are shown in Figure 5(B).

Table 1

Analysis of Vpr-IN complemented and noncomplemented HIV-1 IN mutant viruses

IN mutant viruses	Vpr-IN[a]	RT[b] activity	Virion[c] production	RT:CA ratio	Entry[d]	Infectivity[e]
S-IN	-	103	334	0.31	11.8	13 (0.1)
	+	107	357	0.30	11.6	1896 (15)
H12A	-	457	1016	0.45	10.3	76 (0.6)
	+	368	837	0.44	9.7	3076 (24)
H16A	-	393	929	0.42	10.2	18 (0.1)
	+	378	829	0.45	9.9	2760 (22)
F185A	-	377	868	0.43	46	19 (0.4)
	+	321	784	0.41	10.1	7436 (58)
Δ22	-	415	941	0.44	11.1	62 (0.5)
	+	341	812	0.42	10.6	3974 (31)
D116A	-	401	886	0.45	10.3	1538 (12)
SG3	-	410	934	0.44	10.4	12820 (100)

[a] 4 ug of DNA of each of the viral clones were transfected into 293T cell, either alone (-) or together (+) with 2 ug of the pLR2P-vprIN expression plasmid, using calcium phosphate DNA precipitation methods. Forty-eight hours later, the culture supernatants were harvested, clarified by low-speed centrifugation, filtered through 0.45 micron pore size filters, and saved as stocks.

[b] RT activity (cpm/25 ul, $X10^{-3}$) of culture supernatant virus stocks.

[c] HIV-1 p24 antigen (CA protein) concentration (ng/ml) in culture supernatants. The supernatant stocks were analyzed by HIV-1 antigen ELISA as described by the manufacturer (Coulter Inc.).

[d] Virus entry was quantified by measuring the intracellular HIV-1 CA protein concentration four hours after infection of the HeLa-CD4 cells with 500 ng equivalents (p24 antigen) of each virus stock. The results represent nanograms of p24 antigen per 1 $X10^6$ cells.

[e] Virus infectivity was measured by the MAGI assay as described earlier (32). The infectivity of mutant virus relative to wild-type virus is indicated in parentheses. The wild-type SG3 virus was arbitrarily set to 100.

All values represent the mean of at least 2 independent assays.

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Those skilled in the art will appreciate from the foregoing description that the broad teachings of the present invention can be implemented in a variety of forms. Therefore, while this invention has been described in connection with particular examples thereof, the true scope of the invention should not be so limited since other modifications will be apparent to those skilled in the art upon reviewing the drawings, specification and the appended claims.

All patents and publications cited herein are incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

Claims

1. A method for drug discovery against retroviral replication comprising:

exposing a drug candidate to a monitoring system infected with a retrovirus;

quantifying a retrovirus life cycle function upon exposure of said monitoring system to said drug candidate; and

comparing the retrovirus life cycle function with a retrovirus integrase mutant life cycle function of said monitoring system infected with an integrase mutant retrovirus.

2. The method of claim 1 wherein said retrovirus is HIV-1.

3. The method of claim 1 wherein said monitoring system is a host cell culture.

4. The method of claim 1 wherein said drug candidate is a transgene.

5. The method of claim 1 wherein said drug candidate is selected from a group consisting of: an organic molecule, an organometallic, a polypeptide sequence and a nucleic acid sequence.

6. The method of claim 2 wherein said integrase mutant HIV-1 is selected from a group consisting of:

S-IN, H12A, H16A, F185A, Δ22 and D116A.

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quantifying a retrovirus life cycle function upon exposure of said monitoring system to said drug candidate; and

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3. The method of claim 1 wherein said monitoring system is a host cell culture.

4. The method of claim 1 wherein said drug candidate is a transgene.

5. The method of claim 1 wherein said drug candidate is selected from a group consisting of: an organic molecule, an organometallic, a polypeptide sequence and a nucleic acid sequence.

6. The method of claim 2 wherein said integrase mutant HIV-1 is selected from a group consisting of:

S-IN, H12A, H16A, F185A, Δ22 and D116A.

7. The method of claim 3 wherein said host culture is HeLa-CD4-LTR-β-gal.

8. The method of claim 1 wherein the life cycle function is DNA synthesis.

9. The method of claim 1 wherein the life cycle function is formation of a retroviral RT complex.

10. A method for drug discovery against retroviral replication comprising:

exposing a drug candidate to a monitoring system infected with a retrovirus;

quantifying a retrovirus life cycle function upon exposure of said monitoring system to said drug candidate;

introducing an integrase fusion protein into said monitoring system; and

determining changes in the retroviral life cycle function following introduction of said integrase fusion protein.

11. The method of claim 10 wherein said retrovirus is HIV-1.

12. The method of claim 10 wherein said retrovirus is a mutant.

13. The method of claim 10 wherein said monitoring system is a host cell culture.

14. The method of claim 10 wherein said drug candidate is a transgene.

15. The method of claim 10 wherein said drug candidate is selected from a group consisting of: an organic molecule, an organometallic, a polypeptide sequence and a nucleic acid sequence.

16. The method of claim 10 wherein said integrase fusion protein is an integrase mutant.

17. The method of claim 10 wherein said integrase fusion protein further comprises *vpr*.

18. The method of claim 10 wherein said integrase fusion protein further comprises a polypeptide fragment of *vpr*.

19. The method of claim 10 wherein said integrase fusion protein further comprises *vpx*.

20. The method of claim 10 wherein said integrase fusion protein further comprises a polypeptide fragment of *vpx*.

21. The method of claim 10 wherein said integrase fusion protein is expressed in trans.

22. The method of claim 11 wherein said integrase fusion protein comprises HIV-2 integrase.

23. The method of claim 13 wherein said host cell culture is HeLa-CD4-LTR-β-gal.

24. The method of claim 10 wherein the life cycle function is DNA synthesis.

25. The method of claim 22 wherein the life cycle function is formation of a retroviral RT complex.

26. Use of HIV-1 integrase in preparing a drug for the treatment of HIV infection.

27. A commercial package comprising an integrase fusion according to claim 8 as an active ingredient together with the instructions for the use thereof as a drug candidate screen against retrovirus replication.

28. A commercial package comprising an integrase mutant retrovirus nucleotide sequence according to claim 1 as an active ingredient together with

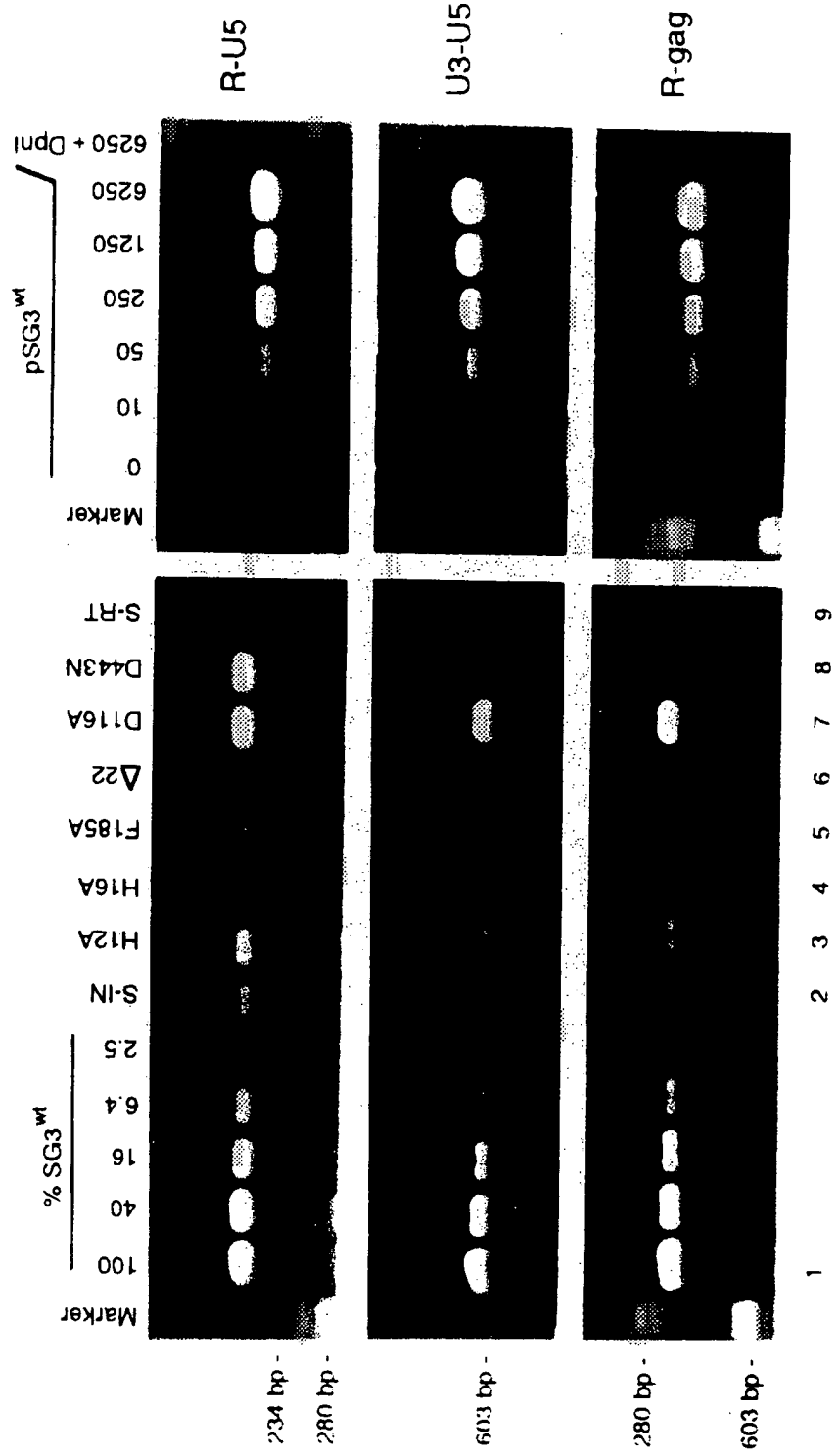

R-U5
U3-U5
R-gag
Marker
0
10
50
250
1250
6250
6250 + DpnI
pSG3wt
Marker
100
40
16
6.4
2.5
% SG3wt
S-IN
H12A
H16A
F185A
Δ22
D116A
D443N
S-RT
1 2 3 4 5 6 7 8 9
234 bp -
280 bp -
603 bp -
280 bp -
603 bp -

FIG-1

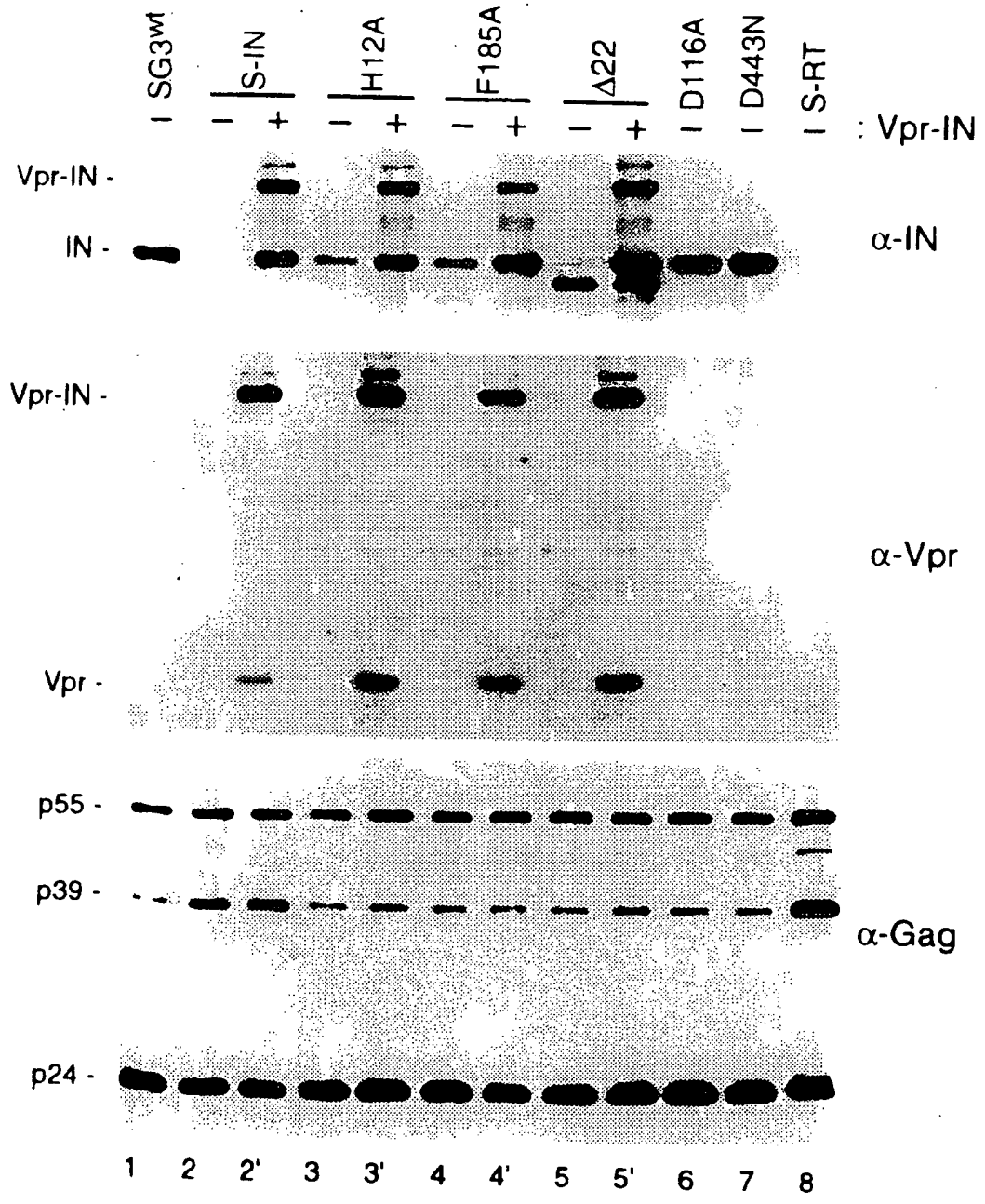

SG3wt
S-IN
H12A
F185A
Δ22
D116A
D443N
S-RT
– + – + – + – +
: Vpr-IN
Vpr-IN -
IN -
α-IN
Vpr-IN -
α-Vpr
Vpr -
p55 -
p39 -
α-Gag
p24 -
1 2 2' 3 3' 4 4' 5 5' 6 7 8

FIG-2A

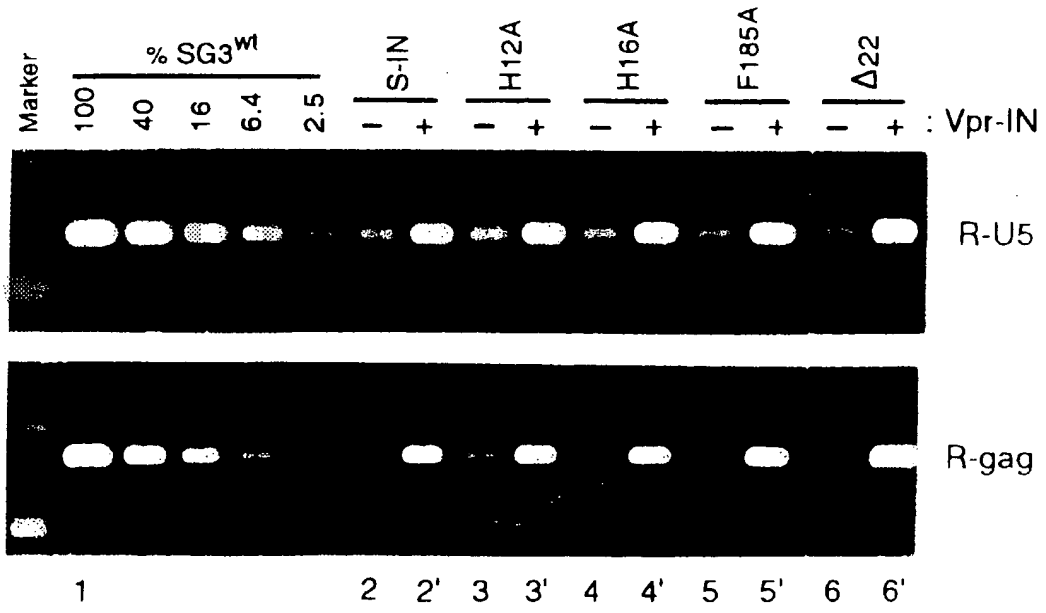

Marker
% SG3wt
100
40
16
6.4
2.5
S-IN
H12A
H16A
F185A
Δ22
– + – + – + – + – +
: Vpr-IN
R-U5
R-gag
1 2 2' 3 3' 4 4' 5 5' 6 6'

FIG-2B

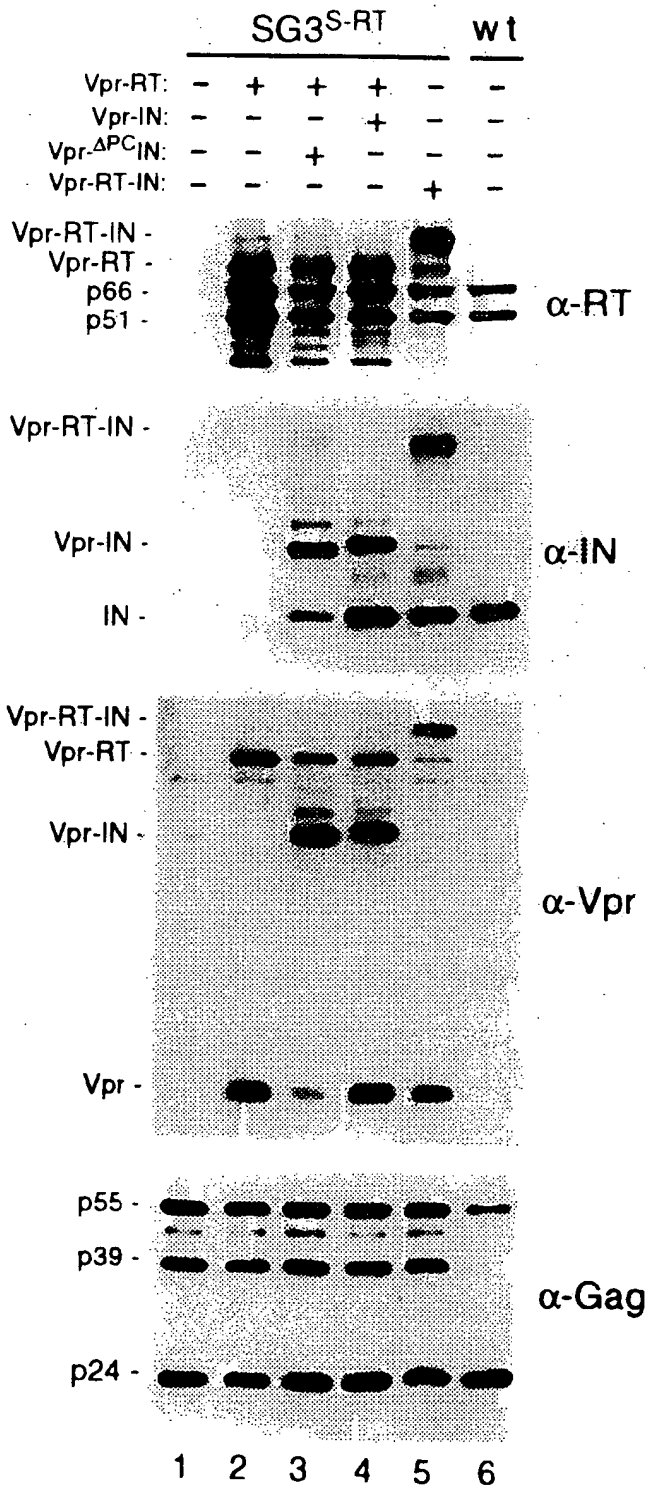

SG3S-RT
wt
Vpr-RT: - + + + - -
Vpr-IN: - - - + - -
Vpr-ΔPCIN: - - + - - -
Vpr-RT-IN: - - - - + -
Vpr-RT-IN -
Vpr-RT -
p66 -
p51 -
α-RT
Vpr-RT-IN -
Vpr-IN -
IN -
α-IN
Vpr-RT-IN -
Vpr-RT -
Vpr-IN -
α-Vpr
Vpr -
p55 -
p39 -
p24 -
α-Gag
1 2 3 4 5 6

FIG-3A

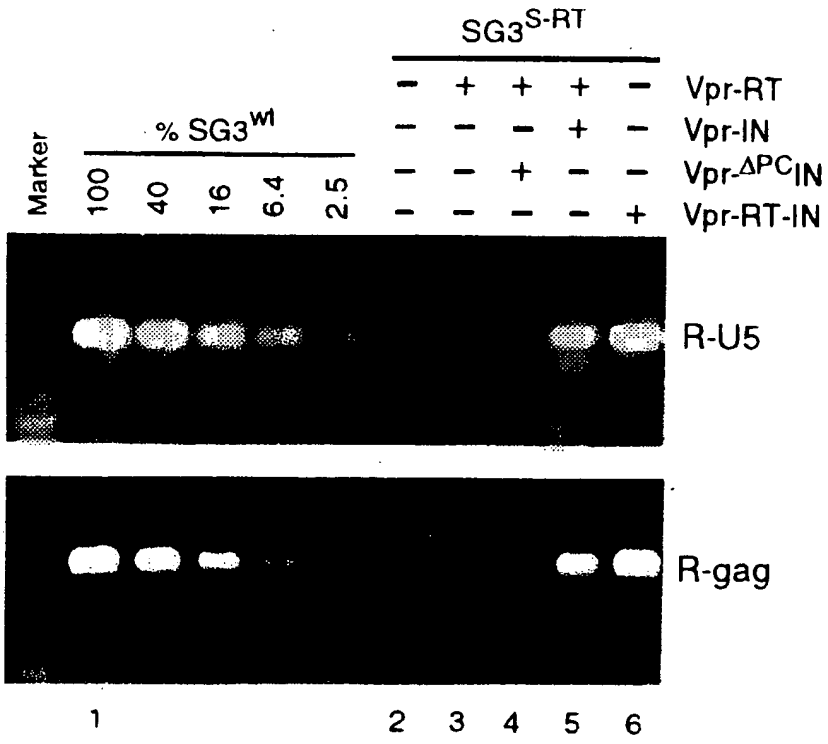
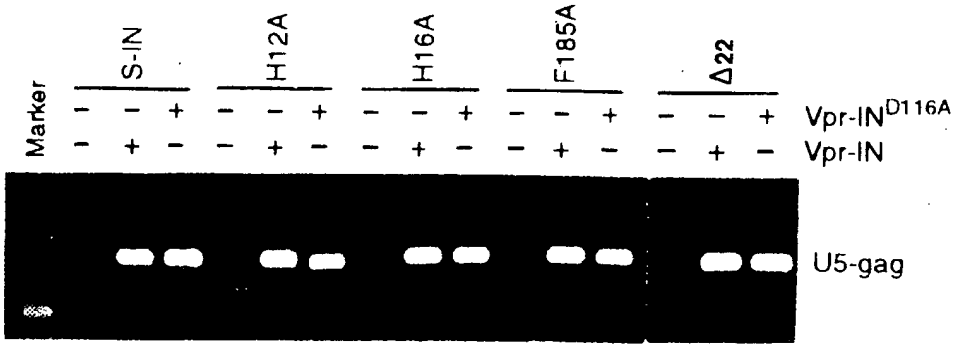

SG3S-RT
Vpr-RT
Vpr-IN
Vpr-ΔPCIN
Vpr-RT-IN
Marker
% SG3WT
100
40
16
6.4
2.5
R-U5
R-gag
1
2
3
4
5
6

FIG-3B

FIG-4A


Marker
S-IN
H12A
H16A
F185A
Δ22
Vpr-IN^{D116A}
Vpr-IN
U5-gag

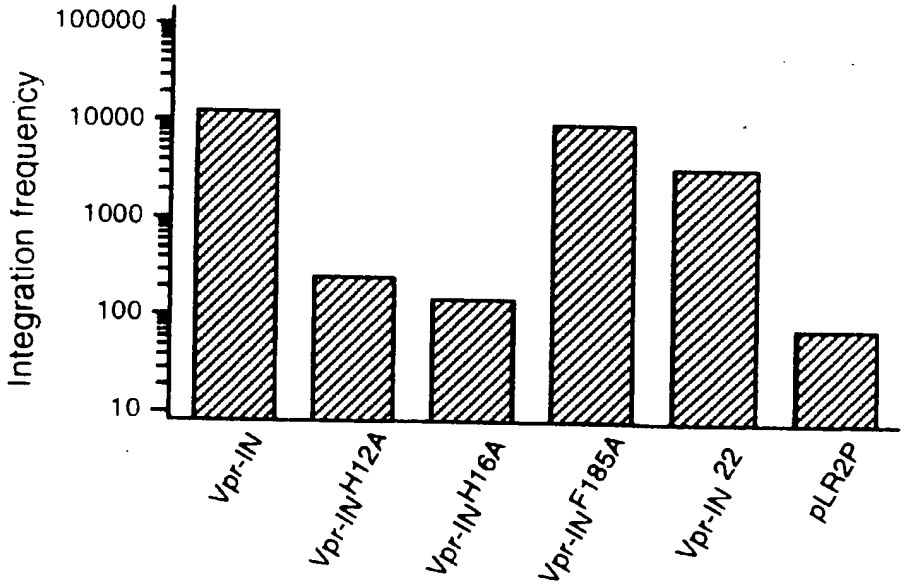
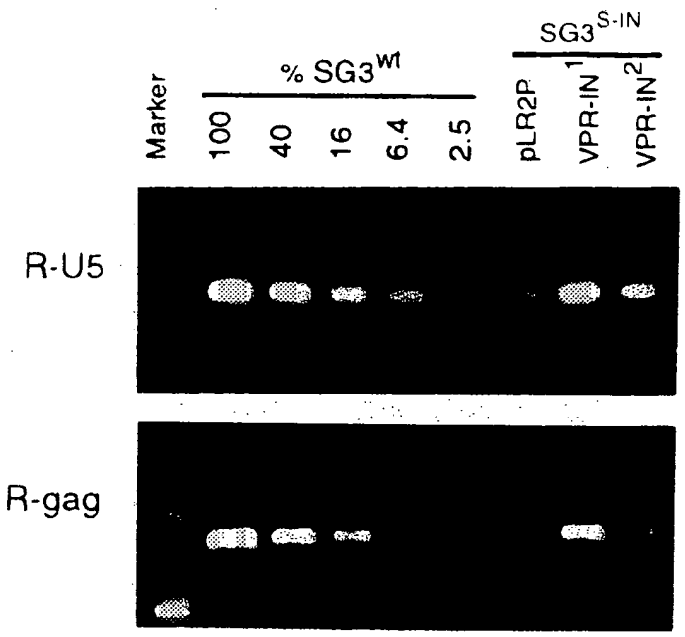

Integration frequency
100000
10000
1000
100
10
Vpr-IN
Vpr-IN/H12A
Vpr-IN/H16A
Vpr-IN/F185A
Vpr-IN 22
pLR2P

FIG-4B

FIG-5A


SG3S-IN
% SG3wt
Marker
100
40
16
6.4
2.5
pLR2P
VPR-IN1
VPR-IN2
R-U5
R-gag

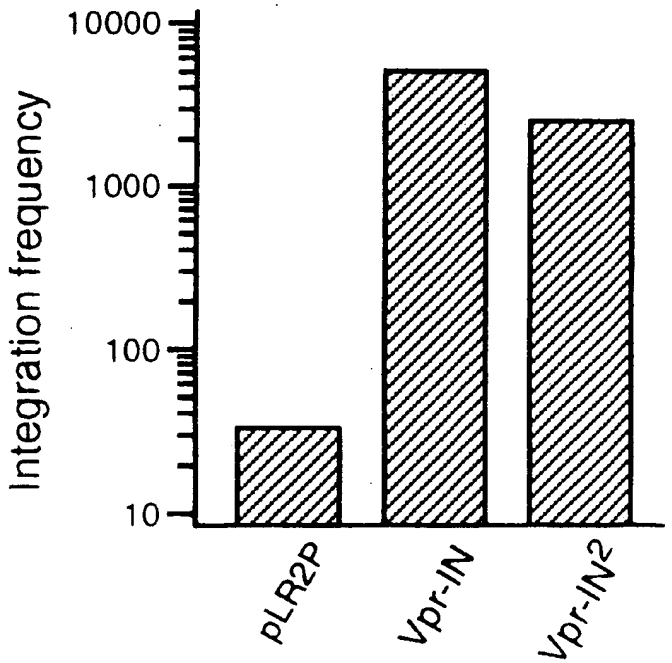

10000
1000
100
10
Integration frequency
pLR2P
Vpr-IN
Vpr-IN2

FIG-5B

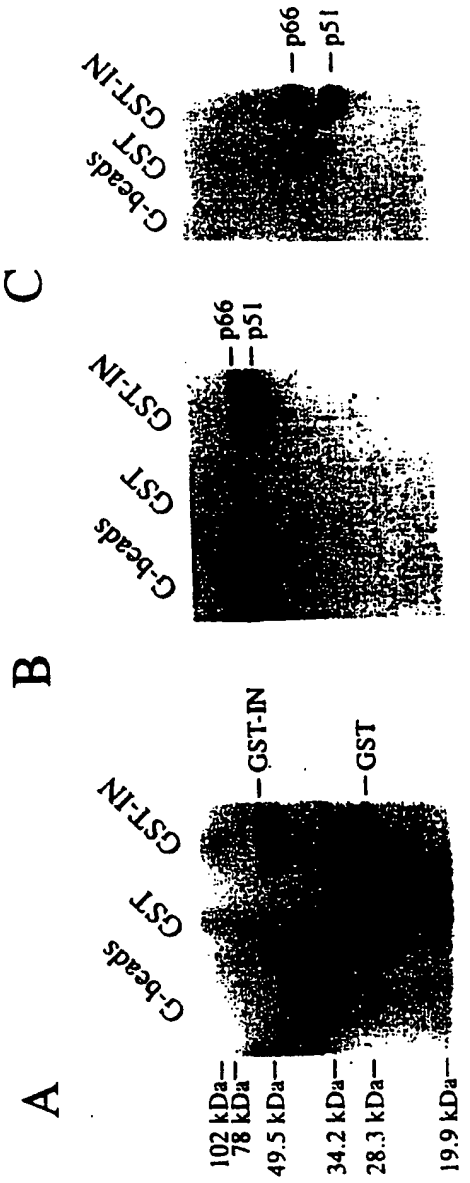

A
B
C
G-beads
GST
GST-IN
GST
GST-IN
102 kDa
78 kDa
49.5 kDa
34.2 kDa
28.3 kDa
19.9 kDa
p66
p51
p66
p51

Figure 6

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: The UAB Research Foundation

(ii) TITLE OF INVENTION: THE USE OF HIV-1 INTEGRASE IN SCREENING HIV-1 DRUG CANDIDATES

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Ellen S. Cogen
Gifford, Krass, Groh, Sprinkle, Anderson & Citkowski, P.C.
(B) STREET: 280 N. Old Woodward Ave., Suite 400
(C) CITY: Birmingham
(D) STATE: Michigan
(E) COUNTRY: U.S.
(F) ZIP: 48009

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM
(C) OPERATING SYSTEM: Windows 95
(D) SOFTWARE: WordPerfect 6.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ellen S. Cogen
(B) REGISTRATION NUMBER: 38,109
(C) REFERENCE/DOCKET NUMBER: ETP-10552/22

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 248-647-6000
(B) TELEFAX: 248-647-5210

(2) INFORMATION FOR SEQ ID NO. 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21
(B) TYPE:
(C) STRANDEDNESS:
(D) TOPOLOGY:

(ii) MOLECULE TYPE:
(A) Description:

(iii) HYPOTHETICAL:

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 1:

GGTCTCTCTG GTTAGACCAG A 21

(3) INFORMATION FOR SEQ ID NO. 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24
(B) TYPE:
(C) STRANDEDNESS:
(D) TOPOLOGY:

(ii) MOLECULE TYPE:
(A) Description:

(iii) HYPOTHETICAL:

(iv) ANTISENSE: Yes

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 2:

CTGCTAGAGA TTTTCCACAC TGAC 24

(4) INFORMATION FOR SEQ ID NO. 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE:
(C) STRANDEDNESS:
(D) TOPOLOGY:

(ii) MOLECULE TYPE:
(A) Description:

(iii) HYPOTHETICAL:

(iv) ANTISENSE: No

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 3:

ACACACAAGG CTACTTCCGT GA 22

(5) INFORMATION FOR SEQ ID NO. 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE:
(C) STRANDEDNESS:
(D) TOPOLOGY:

(ii) MOLECULE TYPE:
(A) Description:

(iii) HYPOTHETICAL:

(iv) ANTISENSE: Yes

(vi) ORIGINAL SOURCE:
(B) STRAIN:
(C) INDIVIDUAL ISOLATE:
(D) DEVELOPMENTAL STAGE:
(F) TISSUE TYPE:
(G) CELL TYPE:
(H) CELL LINE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO. 4:

ATACTGACGC TCTCGCACCC AT 22

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02912

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/70; G01N 33/53, 33/566; C12N 7/00, 15/00

US CL : 435/5, 7.1, 325, 235, 320.1; 436/501

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 7.1, 325, 235, 320.1; 436/501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Aidsline

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAZUMDER et al. Probing Interactions between Viral DNA and Human Immunodeficiency Virus Type 1 Integrase Using Dinucleotides. Molecular Pharmacology. April 1997, Vol.51, No.4, pages 567-575, see entire document.	1-25
X	TADDEO et al. Reversion of a Human Immunodeficiency Virus Type 1 Integrase Mutant at a Second Site Restores Enzyme Function and Virus Infectivity. Journal of Virology. December 1996, Vol.70, No.12, pages 8277-8284, see entire document.	1-25
X	International Conference on AIDS, Vol.11, No.2, 1996, page 217, the abstract No. Th.A.381, FLETCHER III, et al. 'Complementation of Integrase Function in HIV-1 Virions.'	1-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

08 APRIL 1999

Date of mailing of the international search report

11 MAY 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JEFFREY STUCKER

Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02912

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MAZUMDER et al. Effects of Nucleotide Analogues on Human Immunodeficiency Virus Type 1 Integrase. Molecular Pharmacology. April 1996, Vol.49, No.4, pages 621-628, see entire document.	1-25
X	MAZUMDER et al. Effects of Tyrphostins, Protein Kinase Inhibitors, on Human Immunodeficiency Virus Type 1 Integrase. Biochemistry. 21 November 1995, Vol.34, No. 46, pages 15111-15122, see entire document.	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02912

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-25, drawn to methods of screening for retroviral inhibition.
Group II, claim 26, drawn to use of HIV-1 integrase for a drug.
Group III, claim 27, drawn to a kit for integrase fusion.
Group IV, claim 28, drawn to a kit for integrase nucleic acid.
Group V, claim 29, drawn to the integrase protein.
The inventions listed as Groups I-V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The claims lack unity of invention as the inventions of the groups are known in the art, and therefore, the instant claims can not be said to have a special technical feature. Thus, the claims lack unity of invention.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/02912

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-25

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.